Mutation Research 767 (2014) 1–7

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

# Genotoxic evaluation of five Angiotesin II receptor blockers: *In vivo* and *in vitro* micronucleus assay

Iratxe Huerta[a,b], Maitane Barasoain[a], Mercedes Télez[a,b], Mikel Longa[c], Javier Muga[c], Gorka Barrenetxea[d], Eduardo Ortiz-Lastra[d], Javier González[d], Begoña Criado[e], Isabel Arrieta[a,*]

[a] *Department of Genetics, Physical Anthropology and Animal Physiology, Faculty of Science and Technology, University of the Basque Country, Bilbao, Spain*
[b] *Genetic Laboratory, Bilbao, Spain*
[c] *Virgen de Begoña Laboratories (Medikosta), Erandio, Bizkaia, Spain*
[d] *Department of Medical-Surgical Specialties, Faculty of Medicine, University of the Basque Country, Bilbao, Spain*
[e] *Cooperativa de Ensino Superior Politecnico e Universitario (CESPU), Porto, Portugal*

ARTICLE INFO

*Article history:*
Received 15 January 2014
Received in revised form 3 April 2014
Accepted 10 April 2014
Available online 19 April 2014

*Keywords:*
Angiotensin II receptor blockers
Genotoxicity
Micronuclei
FISH

ABSTRACT

Angiotensin II receptor blockers (ARBs) are a new class of drugs for the treatment of hypertension. In this study, we studied the potential genotoxic effects of five ARBs *in vivo* and *in vitro* in human peripheral blood lymphocytes (PBLs) by means of the cytokinesis-block micronucleous (CBMN) assay in combination with fluorescence *in situ* hybridization (FISH) with a centromeric probe. The nuclear division index (NDI) was used as a measure of cytotoxicity. We also analyzed the association between sex, age, duration of treatment and MN formation. The *in vivo* study was carried out in 55 hypertensive patients. The *in vitro* study was performed in 10 control individuals by adding the drugs to the culture medium at a final concentration similar to the levels found in plasma in patients. Our results showed a significant increase in the frequencies of MN and binucleated cells with MN (BNMN) *in vivo* and especially *in vitro*. We observed variability in the mean frequency of MN and BNMN among the five drugs analyzed. *In vivo*, patients treated with Candesartan, Telmisartan and Valsartan showed a statistical significant increase in these parameters, while Olmesartan showed the highest effect *in vitro*. We also found that the drugs inhibit the NDI *in vitro* and that Eprosartan, Olmesartan and Telmisartan are the ARBs studied with the highest effect in decreasing the proliferation of the cells. FISH analysis revealed no significant difference between patients and controls in the frequency of centromeric signals. A slight variability, without statistical significance, in the frequency of micronuclei with a centromere signal ($CN^+MN$) was found among the different ARBs analyzed, ruling out an aneugenic potential. When accounting for risk factors, we found that in patients there is a positive correlation between MN, BNMN and sex and a negative correlation with duration of treatment.



## 1. Introduction

Hypertension is a common treatable condition which confers high risk for coronary artery disease, myocardial infarction, heart failure, stroke, and renal failure [1]. The condition is, therefore, associated with increased cardiovascular morbidity and mortality. The disorder has a prevalence of about 1 billion adults worldwide. The Spanish Society of Hypertension estimates that 35% of the general adult Spanish population suffers from hypertension, of whom 40% are middle aged and 60% are over 60 [2]. Hypertension is commonly treated with antihypertensive drugs, combined with appropriate changes in life-style. The large majority of hypertensive patients need long-term treatment with antihypertensive drugs. Therefore, patients may be exposed to the effects of these drugs over a long period of time.

When prescribing a drug, the benefit/risk ratio is of critical importance [3]. Genotoxic–carcinogenic effects are among the adverse effects that drugs may have, and these should be thoroughly studied. With regards to antihypertensive drugs, epidemiological genotoxicity and carcinogenicity studies show conflicting results, making it difficult to establish whether antihypertensive therapy, while preventing cardiovascular complications, may be associated with an increase in genotoxic–carcinogenic risk [3–5].

* Corresponding author. Tel.: +34 946012605; fax: +34 946013145.
*E-mail address:* mariaisabel.arrieta@ehu.es (I. Arrieta).

http://dx.doi.org/10.1016/j.mrgentox.2014.04.005
1383-5718/

The renin–angiotensin system (RAS) plays an important role in the pathogenesis of various forms of hypertension. Angiotensin II (ANG II), the major effector peptide of the renin–angiotensin system, binds to and activates two receptor subtypes, ANG II type 1 and type 2 ($AT_1$ and $AT_2$), members of the G protein-coupled receptor superfamily (GRCRs), causing oxidative damage. Angiotensin II receptor antagonists or blockers (ARBs) form a class of antihypertensive drugs that block the deleterious effects of ANG II [6].

In 1995, Losartan became the first ARB approved for clinical use, followed by six others, Valsartan, Candesartan, Irbesartan, Telmisartan, Olmesartan and Eprosartan [7]. These drugs have different chemical structures and receptor affinities. According to Miura et al. [8], a small difference in the molecular structure may influence binding affinities. Regarding their chemical structure, two ARBs belong to the non-biphenyl, non-tetrazole class and the other five ARBs belong to the biphenyl-tetrazole class [6,9]. In relation to receptor affinity, some of the drugs compete with ANG II in a concentration-dependent manner by binding to the $AT_1$ receptor (surmountable antagonist), while others are insurmountable antagonists that bind irreversibly to the receptor. Thus, not all ARBs may have the same antihypertensive effects.

A review of genotoxicity and carcinogenicity testing of marketed pharmaceuticals indicates that only three angiotensin II receptor antagonists, Eprosartan, Irbesartan and Telmisartan, have been tested for genotoxicity in human cells [3–5]. In these studies, genotoxicity was assayed in lymphocytes *in vitro* with the chromosomal aberration test. The authors found genotoxic effects in the case of Irbesartan, but not Eprosartan, while the results for Telmisartan were unclear. More recently, a study of cancer incidence after ARB exposure indicated that further investigation is still required [10]. Based on this, a thorough evaluation of the potential genotoxic effects of ARBs in humans is greatly needed.

Human populations exposed to potential genotoxic agents can be monitored through assessment of chemical and biological end-points. Cytogenetic biomarkers are the most frequently used end-point in human biomonitoring studies and are being extensively used to assess the impact of medical factors on genomic stability [11]. The micronucleus (MN) test, standardized by OECD guideline No. 474 [12], is an informative cytogenetic biomarker of early effect, extensively used in genotoxicology and cancer prediction studies [13,14]. This test is usually performed on peripheral blood lymphocytes (PBLs) and has become a standard assay in human biomonitoring studies. The most common method to score MN in cultured lymphocytes is the cytokinesis-block micronucleus (CBNM) assay, where scoring is specifically restricted to cells that have divided once after mitogen stimulation [15,17]. Combination of fluorescence *in situ* hybridization (FISH) using probes for pancentromeric regions with the CBNM assay allows for discrimination between clastogenic (inducing MN containing chromosome fragments) and aneugenic agents (inducing MN containing whole chromosomes) [15,18]. According to Mateuca et al. [19], discriminating between these two phenomena is highly recommended in studies of human genotoxic effects. In addition, basal rates of MN formation in peripheral blood lymphocytes have been shown to be affected by factors such as age, sex, smoking and micronutrient status [11,20–24]. Age and sex are the most important variables [20], critical confounding factors to consider when designing biomonitoring studies [11]. Evaluation of these factors is also important in studies of human genotoxic effects [24].

In previous works, we have studied the genotoxic potential of two antihypertensive drugs, the beta blocker Atenolol and the calcium antagonist Nimodipine [25–27]. Here, we study the genotoxic potential of five Angiotensin II receptor blockers with different chemical structure and receptor affinity characteristics: Candesartan, Eprosartan, Olmesartan, Telmisartan and Valsartan. Eprosartan and Valsartan are surmountable while Candesartan, Olmesartan and Telmisartan are insurmountable antagonists. Eprosartan and Telmisartan belong to the non-biphenyl, non-tetrazole class and Candesartan, Olmesartan and Valsartan belong to the biphenyl-tetrazole class. These five drugs were tested for their ability to induce MN in cultured peripheral lymphocytes of both treated patients and *in vitro* exposed control individuals. The origin of MN was determined by FISH with a probe detecting all human centromeres. The potential association of MN formation with sex, age, duration of treatment was also analyzed.

## 2. Materials and methods

### 2.1. Sample

The sample comprised two groups: 55 hypertensive patients between 49 and 77 years of age (average, 61.6 years), treated by Dr. Ortiz-Lastra (Table 1), and a control group. They were matched by sex, age and smoking habit (in the present study, all individuals were non-smokers). None of them was undergoing other medical treatments. They did not have a previous history of known exposure to genotoxic compounds or a recent X-ray examination. Each patient was given a code (P1-P55) based on the sequence of blood collection. The control group (five males and five females) ranged from 50 to 62 years of age (average, 57.8 years). All were healthy individuals under no medication, with no recent alcohol or drug consumption. Each control individual was given a code (C1-C10) based on the sequence of arrival at the laboratory. All protocols were approved by the Ethics Committee of the University of the Basque Country. Informed consent was obtained for all individuals.

### 2.2. Blood collection and cell cultures

Peripheral blood was collected by venipuncture into sterile vials containing sodium heparin (BD Vacutainer®) and vials were transferred to the laboratory where duplicate whole blood cultures were established within 24 h. For hypertensive patients, the cultures were numbered with the patient code P1-P55. For control individuals, two different cultures were performed: one set numbered C1/T-1-C10/T-1 (control/T-1) and another set where the drug was added to the culture medium at the start of culture. Drugs, kindly provided by Dr. Ortiz-Lastra, had previously been dissolved in distilled water, to give a final concentration in the culture medium that matched the average found in patients after oral intake (these analyses were performed by capillary zone electrophoresis in the Department of Analytical Chemistry, University of the Basque Country, Spain). Data are presented in Table 1. These cultures were numbered C1/medium-C10/medium (controls/medium) (C1/Candesartan-C10/Candesartan, C1/Eprosartan-C10/Eprosartan, C1/Olmesartan-C10/Olmesartan, C1/Telmisartan-C10/Telmisartan and C1/Valdesartan-C10/Valdesartan).

### 2.3. MN assay

Duplicate whole blood cultures were set by adding heparinized blood, 0.5 ml, to RPMI 1640 medium (Gibco®), 4.5 ml, supplemented with 10% fetal bovine serum (Gibco®), antibiotics (penicillin and streptomycin), glutamine, and Hepes buffer solution (Gibco®). Lymphocytes were stimulated with 4% phytohemagglutinin (PHA) (Gibco®). The cultures were incubated at 37 °C for 72 h. Binucleated (BN) cells were accumulated by adding cytochalasin-B (Cyt-B) (Sigma–Aldrich®) at final concentration 6 μg/ml [28], 48 h following initiation of the culture. At the end of the incubation time, the cells were collected by centrifugation. The cells were washed once in RPMI 1640 medium and then a mild hypotonic treatment (2–3 min in 0.075 M KCl at room temperature) was carried out. The cells were then centrifuged and a methanol:acetic acid (5:1) solution was added. This fixation step was performed twice. Air-dried preparations were made and the slides were stained with 10% Giemsa in phosphate buffer for 20 min.

### 2.4. Fluorescence in situ hybridization (FISH)

For the identification of centromeres in MN, FISH was performed with a probe for all human centromeres (QBIOgene®) according to the protocol recommended by the manufacturer. Slides were incubated for 30 min at 37 °C in 2× SSC/0.5% NP-40, pH 7.0 and then dehydrated in a series of ice-cold ethanol washes and dried at room temperature. The probe was applied to slides and cover-slipped. Slides were denatured at 75 °C on a hot plate for 5 min and incubated overnight at 37 °C in a humidified chamber. After hybridization, slides were washed in 1× Wash Buffer (0.5× SSC/0.1% SDS) for 5 min at 65 °C and 1× PBD (phosphate-buffered saline) at room temperature for 5 min. Slides were counterstained with propidium iodide (QBIOgene®). To evaluate probe-hybridization efficiency, metaphase spreads were also examined.